REMARKS

Claims 25-28, 38, 39, 44, 47, 51, 52, 58 and 59 are pending and rejected. Claims 53 and 54 have been cancelled without prejudice and Applicants reserve their right to prosecute subject matter of cancelled claims in subsequent applications. Claim 60 has been added.

Claim 25 has been amended to delete recitation of the phrase "identical or at least 98% sequence similarity" and "having 3'-5' exonuclease activity or". The claim is also amended to correct an obvious error to recite a transgenic plant comprising an endogenous nucleotide sequence encoding a polypeptide of SEQ ID NO:24. SEQ ID NO:23 does not encode a polypeptide. Support for these amendments is in the specification on page 3, lines 17-19.

Claim 44 has been amended to delete recitation of non-elected inventions and to delete the phrase "identical or at least 98% sequence similarity" and "having a 3'-5' exonuclease activity to". Claim 44 has been amended to recite a method of reducing the expression of the endogenous nucleotide sequence encoding a polypepetide of SEQ ID NO:24. Support is in the specification on page 17, lines 6-8; page 31, line 29-page 32, line 10; and claim 44 as originally filed.

Claim 47 has been amended to recite a method for increasing the expression of a nucleotide sequence of interest in a plant cell or plant comprising the steps of:

a) decreasing the expression in said plant cell or plant of an endogenous nucleotide sequence of said plant cell encoding a polypeptide of SEQ ID NO:24 by modifying by insertional mutagenesis in said plant cell at least one chromosomal copy of the nucleotide sequence encoding a polypeptide of SEQ ID NO:24 or of a regulatory region thereof; and

b) introducing into said plant cell or plant a nucleic acid molecule comprising said nucleotide sequence of interest, wherein the expression of said nucleotide sequence of interest in said plant cell or plant is increased. Support for these amendments is in the specification on page 13, lines 10-21.

Claim 51 has been amended to recite a method for increasing the expression of an exogenous nucleotide sequence of interest in a transgenic plant cell or plant comprising the step of:

introducing into said transgenic plant cell or plant of claim 25 an exogenous nucleotide sequence of interest, wherein the expression of said exogenous nucleotide sequence of interest in said transgenic plant cell is increased as

compared to the expression of said nucleotide sequence of interest that was suppressed due to post-transcriptional gene silencing (PTGS) in a plant cell or plant lacking said first expression cassette.

Support for these amendments to claim 51are in the specification on page 33, lines 21-28, page 34, lines 10-11, and page 27, line 28-page 28, line 5.

Claims 52 and 54 have been amended to be dependent upon claim 51.

Claim 53 has been cancelled.

New claim 60 has been added to recite the transgenic plant of claim 25 wherein the endogenous nucleotide sequence encoding the polypeptide of SEQ ID NO:24 is SEQ ID NO:23. Support for this new claim is in the specification on page 8, lines 21-22.

No new matter has been added by these amendments.

Election/Restriction

Claims 51 and 52 as amended are allegedly directed to non-elected inventions and claims 44, 47 and 53 still allegedly contain non-elected subject matter that must be deleted.

In response, Applicants have deleted non-elected subject matter from the claims and claim 53 has been cancelled. These amendments obviate this rejection and Applicants request its withdrawal.

Drawings

Figures 1 and 2 were objected to by the Draftperson. Applicant submit corrected drawings of Figures 1 and 2, thereby overcoming the objections.

Claim Objections

Claims 47, 53 and 54 are objected to for reasons in the Office Action. Claim 47 has been amended to correct the claim, and claims 53 and 54 have been cancelled.

Claim Rejections under 35 USC § 112, second paragraph

Claims 44, 47 and 53 remain rejected under 35 USC § 112, second paragraph as allegedly being indefinite for failing to particularly point out and distinctly claim the invention.

Applicants respectfully disagree, however, claims 44 and 47 have been amended to more particularly point out and distinctly claim the invention and claim 53 has been cancelled.

Claims 25-28, 38, 39, 44, 47, 53, 54, 58 and 59 are rejected under 35 USC § 112, second paragraph for allegedly being indefinite for failing to particularly point out and distinctly claim the invention.

Applicants respectfully disagree, however, claims 25-28, 38, 44, 47, 58 and 59 have been amended to overcome or avoid the rejections. Claims 53 and 54 have been cancelled.

Claim Rejections under 35 USC § 112, first paragraph

Claims 25-28, 38, 39, 44, 47, 53, 54, 58 and 59 are rejected under 35 USC § 112, first paragraph as allegedly containing subject matter that was not described in the specification. Applicants respectfully disagree with these rejections, however, in order to advance prosecution of certain embodiments of the invention, the claims have been amended and claims 53 and 54 have been cancelled.

Claim Rejections under 35 USC §112, first paragraph, enablement

Claims 25-28, 38, 39, 44, 47, 53, 54, 58 and 59 remain rejected under 35 USC §112, first paragraph as allegedly containing subject matter that was not enabled by the specification. Applicants respectfully disagree with this rejection.

Enablement of a disclosure "is not precluded by the necessity for some experimentation such as routine screening." In re Wands, 858 F.2d 731, 736-7 (Fed. Cir. 1988) (citations omitted). The experimentation necessary must not be undue. Id. At 737. Undue experimentation is experimentation that would require a level of ingenuity beyond what is expected from one of ordinary skill in the field. Fields v. Conover, 170 USPQ 276, 279 (CCPA 1971). The factors that can be considered in determining whether an amount of experimentation is undue have been listed in Wands, 858 F.2d at 737. Among these factors are: the amount of effort involved, the guidance provided by the specification, the presence of working examples, the amount of pertinent literature and the level of skill in the art. The test for undue experimentation is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine. Id.

The relevant inquiry for determining whether the scope of the claims is commensurate with the specification is "whether the scope of enablement provided to one of ordinary skill in the art by the disclosure is such as to be commensurate with the scope of protection sought by the claims." In re Moore, 439 F.2d 1232, 1236 (CCPA 1971)

(emphasis added). "A patent need not teach, and preferably omits, what is well known in the art." Hybridtech Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 231 USPQ 81 (Fed. Cir. 1986), cert. Denied, 480 U.S. 947 (1987).

The claims have been amended to delete the recitation of "having 98% sequence similarity and encoding a protein having 3'-5' exonuclease activity" thereby obviating this ground for rejection.

Claims 53 and 54 have been cancelled without prejudice, making this ground for rejection moot.

Regarding the teachings of Examples 5 and 9, Applicants respectfully point out that even though Example 5 was written in the present tense, the results from the work performed in Example 5 are set forth in Table 3 on page 51. Further, Applicants submit herewith a copy of the publication by Glazov *et al.*, The Plant Journal, 35:342-349 (2003) describing the results from the work related to this patent application. In Glazov et al., they isolated a WEX cDNA (AF531179; Genbank sequence provided) which encodes the amino acid sequence of AAO33765.1 (Genbank sequence provided) (corresponding to SEQ ID NO:24 of the present invention). The publication describes the isolation of the sequence; analysis of the T-DNA insertion mutant *wex-1;* characterization of a Post-transcriptional Gene Silencing (PTGS) indicator line; complementation experiments and examination of possible effect of *wex-1* mutation on Transcriptional Gene Silencing (TGS).

The above remarks and published results analyzing the insertional mutant and complementation experiments show the invention is enabled to those skilled in the art.

Regarding the manner in which the claimed method would alter the expression of the endogenous gene and nucleotide of interest, claims have been amended to recite the endogenous nucleotide expression is decreased and that the expression of the nucleotide of interest is decreased. Further, the claims are amended to recite the nucleotide of interest was suppressed due to PTGS before the expression of the endogenous nucleotide sequence was reduced.

These comments and amendments overcome these grounds for rejection and Applicants request their withdrawal.

No fee is believed due, however, please charge any fee to the Deposit Account of Syngenta Biotechnology Inc., #50-1744.

Respectfully submitted,

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A gene encoding an RNase D exonuclease-like protein is required for post-transcriptional silencing in *Arabidopsis*

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Summary

Post-transcriptional gene silencing (PTGS) and the closely related phenomenon RNA interference (RNAi) result from the initial endonucleolytic cleavage of target mRNAs, which are then presumed to be completely hydrolyzed by exoribonucleases. To date, no plant genes required for PTGS are known to encode exoribonucleases. The *Arabidopsis Werner Syndrome-like exonuclease* (*WEX*) gene encodes an RNase D domain most similar to that in human Werner Syndrome protein (WRN), but lacks the RecQ helicase domain. It is also related to *Caenorhabditis elegans mut-7*, which is essential for RNAi, PTGS, and transposon activity. We isolated a loss-of-function mutant, *wex-1*, that showed greatly reduced expression of *WEX* mRNA and early flowering. Although *wex-1* did not affect expression of a robust marker for transcriptional gene silencing (TGS), PTGS of a green-fluorescent-protein (GFP) reporter gene was blocked in *wex-1* and restored by ectopic expression of WEX, indicating that WEX is required for PTGS but not TGS. Thus, members of the RNase D protein family are required for PTGS in both plants and animals. Interestingly, WEX has been shown to interact with an *Arabidopsis* RecQ helicase, suggesting that these proteins might comprise a functional equivalent of WRN.

Keywords: RNA silencing, RNase D exonuclease-like domains, RNA interference, Werner Syndrome protein, PTGS mutants.

Introduction

Post-transcriptional gene silencing (PTGS) and the closely related phenomenon RNA interference (RNAi) are epigenetic forms of mRNA degradation that occur in diverse eukaryotes (Tijsterman *et al.*, 2002). Although genetic and biochemical studies suggest that at least some components of PTGS/RNAi have been highly conserved in evolution, features such as transitivity, systemic spread, and downstream steps in RNA degradation can differ in different organisms (Sijen *et al.*, 2001; Tijsterman *et al.*, 2002; Vaistij *et al.*, 2002). In addition, there is growing evidence for links between PTGS/RNAi and transcriptional gene silencing (TGS), which is accompanied by methylation of cytosines in the promoter region of the target genes (Aufsatz *et al.*, 2002; Vaucheret *et al.*, 2001). At present, it is not clear if multiple mechanisms for PTGS exist and to what extent these mechanisms and those for TGS overlap at the molecular level.

PTGS in plants, like RNAi in animals, is probably initiated by double-stranded RNAs (dsRNAs) that are processed by Dicer-like RNase III activity to small interfering RNA (siRNA) duplexes, which guide the endonucleolytic cleavage of cognate RNAs in the region of complementarity (Matzke *et al.*, 2001; Plasterk, 2002; Tang *et al.*, 2003; Vaucheret *et al.*, 2001). The resultant fragments are then completely hydrolyzed, presumably by exoribonucleases. Previous genetic and molecular studies of *Arabidopsis* have identified four genes necessary for PTGS: the *SGS2/SDE1* gene encoding a putative RNA-dependent RNA polymerase (RdRP), which may help synthesize dsRNAs (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000; Vaistij *et al.*, 2002); the *SDE3* gene encoding an RNA helicase (Dalmay *et al.*, 2001); the *AGO1* encoding a PAZ-domain protein (Cerutti *et al.*, 2000; Fagard *et al.*, 2000); and the *SGS3* gene encoding a plant-specific protein of unknown function with

coiled-coil domains (Mourrain *et al.*, 2000). In addition, the *SIN1/SUS1/CAF* (DCL1) gene, which encodes a Dicer-like protein (Schauer *et al.*, 2002), has been shown to be important for the formation of micro RNAs from dsRNA (Golden *et al.*, 2002; Reinhard *et al.*, 2002). To date, no genes encoding exoribonucleases have been implicated in plant PTGS.

The *Caenorhabditis elegans mut-7* gene has been shown to be essential for RNAi, PTGS, and silencing of transposon activity (Ketting *et al.*, 1999; Tabara *et al.*, 1999). It encodes a member of the RNase D protein family belonging to the DEDD superfamily of 3′-5′ exoribonucleases (Mian, 1997; Zuo and Deutscher, 2001). Some members of this family are important for processing and maturation of small RNA species such as tRNAs and snRNAs in *Escherichia coli* and yeast (Zuo and Deutscher, 2001). Thus, MUT-7 may function in the degradation of mRNAs targeted by PTGS or RNAi. The MUT-7 protein is similar to the human Werner Syndrome protein (WRN) (Yu *et al.*, 1996), which has been implicated in premature aging and contains functional RecQ helicase and RNase D domains (Shen and Loeb, 2000; Shen *et al.*, 1998).

Here, we show that the *Arabidopsis Werner Syndrome-like exonuclease* (*WEX*) gene, which encodes an RNase D protein related to WRN and MUT-7 is required for PTGS, but not for TGS.

Results

Identification of Arabidopsis *RNase D family members*

The *C. elegans* MUT-7 protein is the only member of the RNase D family known to be implicated in PTGS. We used BLASTP (Altschul *et al.*, 1990) and Hidden Markov Model (HMM) searches (Eddy, 1996) performed with the 3′-5′ exonuclease Pfam profile (01612) (Bateman *et al.*, 2000) to find *Arabidopsis* homologs with a similar role in plant PTGS. We identified six proteins with potential RNase D domains (HMM *E*-values $<1e^{-10}$): AAD25623, AAC69936, CAB36851, AAC42241, AAG50917, and BAB11227 (Table S1). These proteins show sequence conservation in the three Exo motifs that cluster around the active site of the RNase D domain with the five most conserved residues present in all the proteins, except for the tyrosine in AAC42241 (Mian, 1997). CAB36851 was most closely related to MUT-7 (BLASTP *E*-value = $7e^{-4}$) and had an RNase D-like domain most similar to that in human WRN. We call the gene encoding CAB36851, previously designated *AtWRNexo* (Hartung *et al.*, 2000), *WEX* to comply with the accepted nomenclature for *Arabidopsis* genes.

We isolated a *WEX* cDNA (AF531179) by rapid amplification of cDNA ends (RACE) and found that the WEX protein differs from that predicted in CAB36851. All twelve 3′ RACE RT-PCR products examined for this cDNA have an additional nine bases (869–877) not present in the published cDNA (AJ404476) (Hartung *et al.*, 2000), suggestive of alternative splicing. A closely related rice gene produces two transcripts differing by 9 bp because of alternative 3′ splice sites at the same position in the protein (J.Z.L., unpublished data). This portion of the WEX protein is predicted to be part of an alpha helix in the Exo III motif close to the C-terminus of the protein (Mian, 1997). Examination of EST sequences in GenBank provides evidence for what appears to be an expressed pseudogene with sequence similarity to *WEX* upstream of the *WEX* gene (see Figure S1).

The T-DNA insertion mutant wex-1 *shows greatly reduced* WEX *mRNA expression*

We obtained the loss-of-function *wex-1* mutant by PCR screening of a large pool of T-DNA lines (Krysan *et al.*, 1999). This mutant contains a T-DNA insertion in the 5′-UTR 26 bp upstream of the start codon. Figure 1 shows that *WEX* mRNA expression of *wex-1* plants was greatly reduced relative to the wild type. *WEX* mRNA could be detected by RNA blot hybridization of poly(A)$^+$ RNA from wild-type plants but not *wex-1* plants (Figure 1a). The *WEX* RT-PCR product was readily detectable with

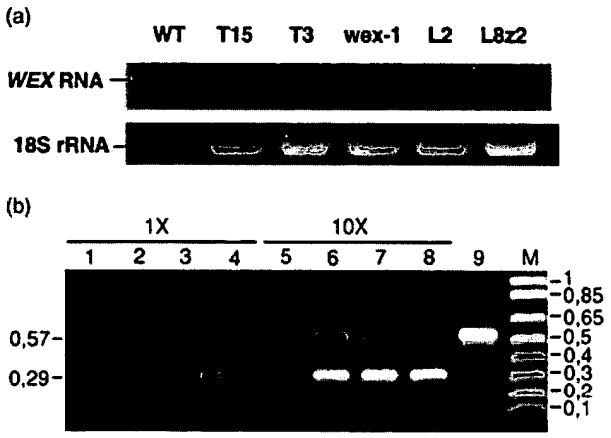

Figure 1. *wex-1* shows greatly reduced *Werner Syndrome-like exonuclease* (*WEX*) mRNA expression.
(a) RNA blot hybridization of poly(A)$^+$ RNA prepared from wild type (WT), *wex-1/wex-1 35S$_2$-GFP/35S$_2$-GFP* (T15), *WEX/WEX 35S$_2$-GFP/35S$_2$-GFP* (T3), *wex-1/wex-1* (*wex-1*), line 2 (L2), and line 8z2 (L8z2) plants using *WEX* cDNA as a hybridization probe. The 18s rRNA used as a loading standard was stained with ethidium bromide.
(b) RT-PCR with cDNAs amplified for 30 PCR cycles. A 0.8 % agarose gel was loaded with 10 μl of the PCR reaction mixture (10×) or a 1/10 dilution of this mixture (1×) obtained with *wex-1* cDNA (lanes 1 and 5), wild-type cDNA (lanes 2 and 6), line 8z2 cDNA (lanes 3 and 7), line 2 cDNA (lanes 4 and 8), and wild-type genomic DNA (lane 9). Gels were stained with ethidium bromide. The sizes of the molecular weight markers (lane M) in kb are indicated on the right; the sizes of the amplification products on the left. The PCR product amplified using genomic DNA gave the 570-bp product expected for the *WEX* primers used.

total RNA from wild-type plants, but only barely detectable with total RNA from the *wex-1* mutant, even when 10-fold more of the reaction mixture was loaded on the gel (Figure 1b).

Although *wex-1* plants showed greatly reduced levels of *WEX* mRNA, they did not show visible abnormalities and were morphologically indistinguishable from the wild type at the embryo, seedling, and flowering stages (Figure 2a,e and data not shown). Interestingly, preliminary data suggest that, under our standard conditions (16-h light/8-h darkness, 21°C, 65% humidity), *wex-1* plants flowered 10–15 days earlier than the wild type, which flowered after 40–50 days (Figure 2a,b).

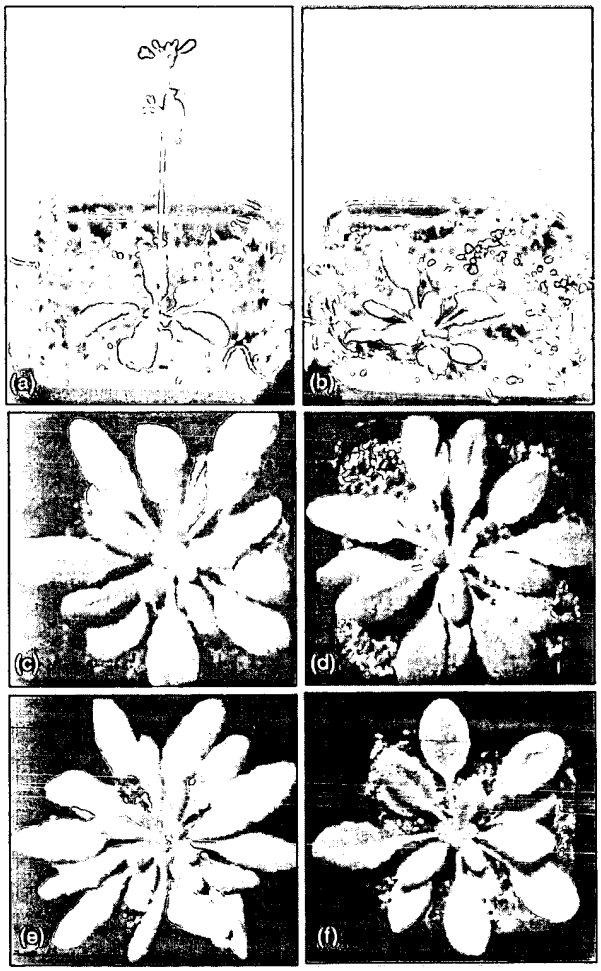

Figure 2. Expression of green fluorescent protein (GFP) in representative $35S_2$-GFP transformants.
(a,b) *wex-1* mutant (a) and wild type (b) plants illuminated with white light and photographed 7 days after germination.
(c–f) Representative line 2 plants, which show stable, high-level GFP expression (c), silent 8z2 plants (d), F_3 generation T15 *wex-1*/*wex-1* $35S_2$-GFP/ $35S_2$-GFP plants (e), and wild-type, untransformed plants (f) photographed at rosette stage under UV illumination. Plants illuminated by UV light exhibit green fluorescence when GFP is expressed and red chlorophyll fluorescence when GFP expression is silenced.

Characterization of a PTGS indicator line

To assay for effects of *wex-1* on PTGS, we generated the silent indicator line 8z2. This line and the high-green-fluorescent-protein (GFP)-expressing line 2, which served as a positive control, are independent, monogenic lines homozygous for a chimeric *GFP* reporter gene regulated by a duplicated cauliflower mosaic virus 35S RNA promoter ($35S_2$-GFP). We partially characterized the T-DNA inserts by Southern blot hybridization (Figure S2) and mapped their positions in the *Arabidopsis* genome by thermal asymmetric interlaced PCR (TAIL-PCR). Line 8z2 contains a T-DNA direct tandem repeat inserted at position 42963 in genomic clone F22L4 on chromosome 1 (AC061957), which was confirmed by sequencing the entire locus. Line 2 contains a single T-DNA insert with its left border at position 46355 in genomic clone T16F16 (AC005312) on chromosome 2.

Control line 2 plants showed uniform, high-level GFP expression at rosette stage (Figure 2c) and throughout sporophytic development in more than 500 plants scored (data not shown). Line 8z2 plants exhibited resetting of silencing, which is characteristic of PTGS (Meins, 2000), i.e., cotyledon-stage seedling were high-GFP expressing. Silencing started at the two-leaf stage, which was evident in essentially all visible parts of the plants by mid-rosette stage (Figure 2d) and then persisted throughout sporophytic development (data not shown). Of more than 500 plants scored in several experiments, the incidence of silencing at mid-rosette stage ranged from 90–100%. No silencing was detected in plants hemizygous for the *GFP* transgene (data not shown). RNA blot hybridization confirmed a marked reduction of *GFP* mRNA in silent line 8z2 plants relative to that of high-GFP-expressing line 2 plants (Figure 3a). Comparison of these steady-state mRNA levels with those of nascent *GFP* RNA determined by nuclear run-on transcription measurements (Figure 3b) showed that silencing of GFP expression in line 8z2 results from PTGS rather than from a decreased rate of transcription. This conclusion was confirmed by the finding that small, *c.* 21–25 nt long RNAs (smRNAs), considered diagnostic for PTGS (Hamilton and Baulcombe, 1999), accumulated in silent line 8z2 tissues, but not in high-GFP-expressing line 2 tissues (Figure 4).

WEX expression is required for PTGS, but not for TGS

We obtained plants homozygous for *wex-1* and for the *$35S_2$-GFP* T-DNA by crossing the *wex-1* mutant with line 8z2 and PCR genotyping the F_2 generation with gene-specific primers that span the insertion sites. Populations of *wex-1*/*wex-1* and *WEX*/*WEX* plants in the F_3 generation and parental 8z2 plants were scored for incidence of PTGS. Measurements of *WEX* mRNA confirmed that neither the presence of the GFP transgene nor silencing of this gene

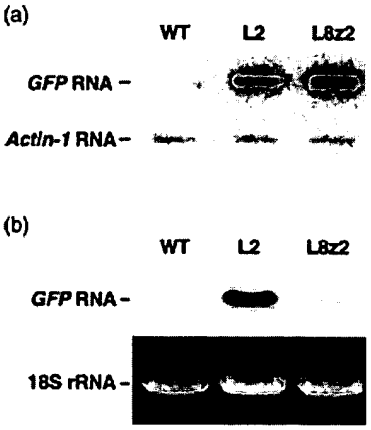

Figure 3. Silencing of green fluorescent protein (*GFP*) mRNA accumulation in line 8z2 is at the post-transcriptional level.
(a) Nuclear run-on transcription measurements of *GFP* RNA using *Arabidopsis actin-1* RNA as a control and nuclei prepared from wild-type (WT, untransformed), line 2 (L2), and line 8z2 (L8z2) leaves.
(b) RNA blot hybridization with a probe for *GFP* RNA of poly(A)$^+$ RNA prepared from the same pooled tissues used in the nuclear run-on transcription experiment shown in (a).

had appreciable effects on expression of wild-type *WEX* and *wex-1* alleles (Figure 1a,b).

The *wex-1* mutation strongly inhibited GFP PTGS. Plants homozygous for both the $35S_2$-GFP locus and *wex-1*, which had greatly reduced *WEX* mRNA contents relative to untransformed *WEX* plants (Figure 1a,b), showed high levels of GFP fluorescence (Figure 2c,e) and *GFP* mRNA accumulation (Figure 4a) comparable to the line 2 control, and, like the high-GFP-expressing line 2, did not accumulate *GFP* smRNAs (Figure 4b). None of the 36 plants scored exhibited PTGS, which is significantly different from the high incidence found with the parental 8z2 line or the outcrossed line carrying wild-type *WEX* allele (Table 1).

Table 1 Effect of the *wex-1* mutation on the incidence of *$35S_2$-GFP* post-transcriptional gene silencing (PTGS)

Line	Description	Incidence of PTGS (%)*
8z2	P_1 Generation *WEX/WEX 35S$_2$-GFP/35S$_2$-GFP*	90 (40)[a]
T3	F_3 Generation *WEX/WEX 35S$_2$-GFP/35S$_2$-GFP*	88 (33)[b]
T15	F_3 Generation *wex-1/wex-1 35S$_2$-GFP/35S$_2$-GFP*	0 (36)[a,b,c]
TW	*Complemented T15 wex-1/wex-1 35S$_2$-GFP/35S$_2$-GFP + Ubq3-WEX cDNA*	92 (27)[c]

*Percentage of the total number of plants scored shown in parenthesis exhibiting a silent phenotype at rosette stage. Values with the same letter are significantly different ($P < 0.0005$, Binomial Proportions Test).

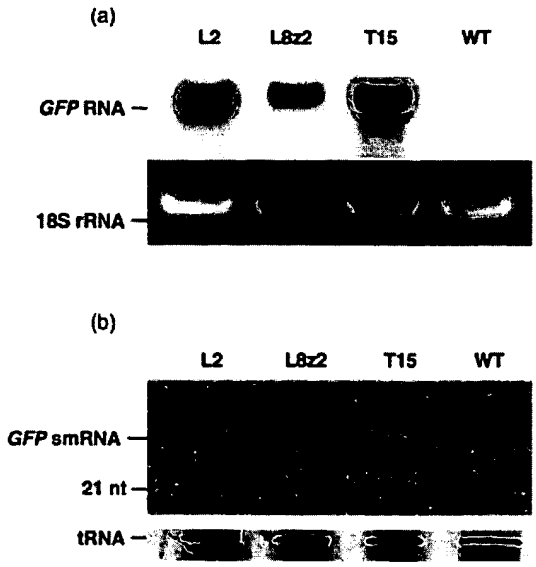

Figure 4. Accumulation of green fluorescent protein (*GFP*) smRNAs is associated with GFP post-transcriptional gene silencing (PTGS).
(a,b) RNA blot hybridization of high- and low-molecular-weight fractions of total RNA prepared from line 2 (L2), line 8z2 (L8z2), *wex-1/wex-1 35S$_2$-GFP/35S$_2$-GFP* (T15) and wild-type, untransformed (WT) leaf tissue. Loading standards, 18S rRNA and a selected tRNA as indicated, were stained with ethidium bromide. (a) High-molecular-weight fraction hybridized with a probe for *GFP* mRNA. (b) Low-molecular-weight fraction hybridized with a probe for sense *GFP* RNA. The position of the 21 nt oligoribonucleotide size standard is indicated.

Complementation experiments confirmed that *WEX* expression is required for PTGS. We selected monogenic, homozygous Ubq3-WEX transformants carrying the *WEX* cDNA and a hygromycin resistance marker that expressed appreciably higher *WEX* mRNA levels than the wild type (data not shown). These lines were crossed with line T15 (*wex-1/wex-1 35S$_2$-GFP/35S$_2$-GFP*; Table 1), and the F_2 generation was screened for hygromycin-resistant *wex-1/wex-1 35S$_2$-GFP/35S$_2$-GFP* plants. PTGS was restored in *wex-1/wex-1* plants carrying at least one copy of the Ubq3-WEX transgene. The incidence of GFP silencing in the complemented line TW, 92%, was significantly higher than that in the *wex-1/wex-1* line, but did not differ significantly from the incidence of silencing in *WEX/WEX* lines (Table 1).

To examine possible effects of the *wex-1* mutation on TGS, we measured expression of transcriptionally silent information (TSI) RNAs, which have been shown to be robust markers for TGS (Steimer *et al.*, 2000). These RNAs are expressed in several TGS-defective mutants, including *mom1*, but not in the wild type. RNA blot hybridization showed that the two RNAs detected with a probe for TSI-B RNA are expressed in *mom1*, but not in wild-type or *wex-1* plants (data not shown). Thus, WEX does not appear to be required for TGS.

Discussion

RNase D protein family members are required for PTGS in both plants and animals

Our results, together with recent studies of the *C. elegans mut-7* mutant (Tabara *et al.*, 1999), show that members of the RNase D protein family are required for PTGS in both plants and animals. *wex-1* plants show greatly reduced accumulation of WEX mRNA and are deficient in PTGS, which is restored by ectopic expression of a *WEX* transgene. This strongly suggests that *wex-1* is a recessive, loss-of-function mutant, and that the *WEX* gene product is required for PTGS. The *C. elegans mut-7* mutant shows heritable parent-of-origin effects, which are often associated with TGS in other organisms (Reik *et al.* 2001), and frequently loses its X chromosome during meiosis (Ketting *et al.*, 1999). Mutations in the human *WRN* gene lead to similar genetic instabilities and premature onset of age-related diseases (Shen and Loeb, 2000). By contrast, the *wex-1* mutant did not show obvious effects on the growth or development of *Arabidopsis* other than early flowering. Although decreased DNA methylation can result in early flowering under short-day conditions (Sheldon *et al.*, 1999)TGS was not blocked in *wex-1*.

Interacting WEX and RecQ helicases in Arabidopsis *could be the functional equivalent of WRN*

The domain structures of several WEX-related proteins are shown in Figure 5. *Arabidopsis* WEX and *C. elegans* MUT-7 are closely related to human WRN in the RNase D family, but lack a RecQ helicase domain. Interestingly, the *Neurospora* QDE3 protein, which is required for PTGS, has a RecQ helicase domain, but no RNase D domain (Cogoni and Macino, 1999). Six members of the RecQ helicase family have been identified in Arabidopsis (Hartung et al., 2000). One of these, AtRecQI2, has been shown to interact with WEX in a yeast two-hybrid system. Together, these findings lead us to speculate that, in *Arabidopsis*, an interacting RecQ helicase and RNase D domain protein could be the functional equivalent of WRN. It is not known if both domains are required for PTGS.

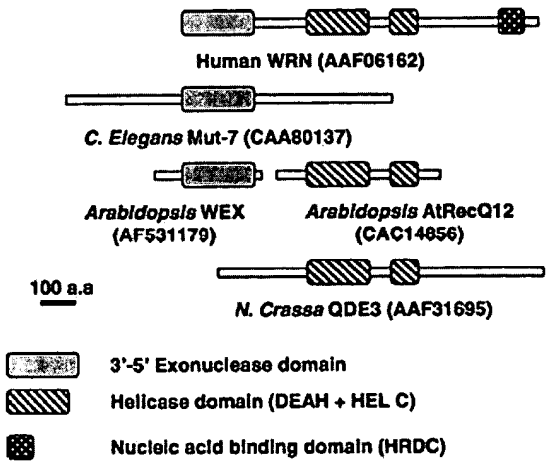

Figure 5. Schematic domain structure of Werner Syndrome protein (WRN)-like proteins.
Accession numbers are indicated in parentheses. Sizes of proteins and domains in amino acid residues are approximate. We speculate that two proteins in *Arabidopsis*, Werner Syndrome-like exonuclease (WEX), and AtRecQI2, which are binding partners, could be the functional equivalent of WRN in humans.

Where does WEX act in PTGS pathway

Werner Syndrome-like exonuclease is a member of the DEDD superfamily of 3′-5′ exoribonucleases, which includes proteins important for RNA processing and maturation (van Hoof and Parker, 1999; Mian, 1997; Zuo and Deutscher, 2001). WEX, however, does not have an essential housekeeping function because the *wex-1* mutant is viable and develops normally. WEX appears to have PTGS-specific functions. Where and how WEX acts in the PTGS pathway is not known. Considerable evidence suggests that plant siRNAs guide the degradation of cognate RNAs in an RISC-like endonuclease complex (Tang *et al.*, 2003). This involves an initial endonucleolytic cleavage and subsequent hydrolysis of target RNAs, presumably mediated by 3′-5′ and 5′-3′ exoribonucleases (Matzke *et al.*, 2001; Meins, 2000). Thus, WEX could specifically degrade the products of endonuclease activity. This hypothesis is difficult to reconcile with our finding that WEX is required for PTGS, as the initial endonucleolytic cleavage of the RNA should be sufficient to impair its translational activity. Nevertheless, *GFP* mRNA levels, as well as GFP expression, were restored in the *wex-1* mutant, and no truncated *GFP* mRNAs, likely to form if degradation were blocked after initial endonucleolytic cleavage, were detected (data not shown). Two possible explanations are that WEX is an essential component of the RISC-like endonuclease complex, or that the removal of endonuclease products from the complex is required for siRNA-guided RNA degradation. Another possibility, which we favor, is that WEX acts upstream of RISC. This working hypothesis is consistent with our finding that smRNAs, presumably generated by Dicer-like activity, accumulate in silent WEX/WEXGFP plants, but not in wex-*1*/*wex-1* GFP plants impaired in *GFP* RNA silencing. Finally, we cannot rule out the possibility that WEX acts at more than one site, e.g. upstream and downstream of siRNA formation, as has been proposed for putative RNA-dependent RNA polymerases (Dalmay *et al.*, 2000; Klahre *et al.*, 2002; Mourrain *et al.*, 2000; Vaistij *et al.*, 2002).

Experimental procedures

Isolation of a WEX *cDNA*

Primers for 5′ and 3′ RACE were designed based on the exon/intron boundaries for the predicted protein (CAB36851). PCR products from 5′ and 3′ RACE (GeneRacer kit, Invitrogen, Basel, Switzerland) were TA-cloned (Original TA-Cloning kit, Invitrogen), and the longest clones were assembled to form an 1150-bp *WEX* cDNA sequence (AF531179).

Isolation of the wex-1 *mutant*

A pool of approximately 60 480 independent T-DNA lines of *Arabidopsis* ecotype Wassilewskija (Krysan *et al.*, 1999) was screened by PCR, utilizing primers corresponding to the pD991 T-DNA left border and the *WEX* 3′-specific region. These lines carry an *nptII* gene, which confers kanamycin resistance, regulated by the *mas* promoter and transcriptional terminator. De-convolution of pool architecture and genotyping by PCR led to the identification of a line homozygous for the insertion, which was designated *wex-1*.

Plant material

Unless indicated otherwise *Arabidopsis thaliana* ecotype Columbia plants were used. Transgenic plants were obtained by *Agrobacterium*-mediated transformation (Bechtold and Pelletier, 1998) with two Ti-plasmids. p35S_2-GFP carries a chimeric GFP (Reichel *et al.*, 1996) reporter gene regulated by a duplicated cauliflower mosaic virus 35S RNA promoter and transcriptional terminator (Goodall and Filipowicz, 1989) and an *nptII* gene regulated by the *nos* promoter and transcriptional terminator in binary vector pBIN19 (Bevan, 1984). pUbq3-WEX carries the coding region of *WEX* cDNA regulated by the *Arabidopsis ubiquitin 3* promoter (Norris *et al.*, 1993) and *nos* transcriptional terminator and an *hptII* gene, which confers hygromycin resistance, regulated by a duplicated cauliflower mosaic virus 35S RNA promoter and transcriptional terminator in binary vector pCAMBIA-1380 (CAMBIA, Canberra, Australia). Monogenic transformants homozygous for the resistance markers were obtained from the F_2 generation of selfed transformants.

Assaying for GFP fluorescence

GFP fluorescence was monitored by illuminating plants with UV light using a 'BLAK-RAY®' semiconductor inspection lamp (Omnilab, Mettmenstetten, Switzerland).

DNA analysis

Total genomic DNA was extracted from leaf tissue using the Nucleon PhytoPure DNA extraction kit (Amersham Biosciences, Dübendorf, Switzerland). Southern blot hybridization was performed with digoxigenin (DIG)-labeled DNA probes synthesized using a PCR DIG Probe Synthesis Kit (Roche Diagnostics, Rotkreuz, Switzerland). Primers for amplification of *GFP* and *nptII* probes were GFP1 (5′-AAGGAAGAGGAGCTCTTCACCG), GFP2 (5′-TTCTGCTGGTAGTGGTCGGC), NPTII1 (5′-CGCATGATTGAACAAGATGG), and NPTII2 (5′-AACGATTCCGAAGCCCAAC).

The genomic position of the T-DNAs was determined by TAIL-PCR (Liu *et al.*, 1995) using T-DNA-specific primers LB1 (5′-TTCGGAACCACCATCAAACAGG), LB2 (5′-TTGCTGCAACTCTCTCAGGGCC), LB3 (5′-TCAGCTGTTGCCCGTCTCACT), and the degenerate primer AD3 (5′-WGTGNAGWANCANAGA, where W=A/T and N=G/A/T/C). Amplified PCR fragments were ligated into pGEM-T Easy vector (Promega, Wallisellen, Switzerland), sequenced, and used as a query in BLAST searches to identify genomic DNA adjacent to a T-DNA border. Transformants were genotyped using the T-DNA-specific primer 5′-CATTTTATAATAACGCTGCGGACATCTAC and the *WEX* gene-specific primer 5′-CGACATGATCTGATACATCGTTATGCCAAT to detect the insertion in *WEX*; the T-DNA-specific primer LB1 and gene-specific primers 5′-TTCGAAAACATTACCTCCGATC and 5′-GGCTTTTGCATTTGGTATCTACTAG were used to detect the 35S_2-GFP T-DNA insertion.

RNA analysis

Total RNA was extracted from leaf tissue using Trizol reagent (Gibco BRL). Poly(A)+ RNA was isolated using a Poly(A) Pure mRNA purification kit (Ambion, Huntingdon, UK). High-molecular-weight and low-molecular-weight RNA fractions of the same total RNA preparation were obtained using an RNeasy Mini Kit (Qiagen, Basel, Switzerland) as modified by Di Serio *et al.* (2001). For RNA blot hybridization, 10-μg aliquots of RNA were loaded onto a 0.8% (w/v) denaturing agarose gel containing 50% (v/v) of formaldehyde. RNA blotting and hybridization of high-molecular-weight RNAs were carried out using DIG-labeled DNA probes (Roche). The hybridization probes used to detect sense *GFP* smRNAs were a mixture of ^{32}P end-labeled oligodeoxynucleotides covering the *GFP* coding region. TSI RNA was measured with a probe for TSI-B as described by Steimer *et al.* (2000). For RT-PCR, cDNA was synthesized using Omniscript Reverse Transcriptase (Qiagen), utilizing 1 μg of total RNA as a template for the randomly primed reverse transcription reaction. cDNAs obtained from the reverse transcription reaction were used for subsequent PCR amplification of *WEX*. The primers WEX CDS_F (5′-ATGTCATCGTCAAATTGGATCGACG) and WEX_R (5′-CGCTTATCAACCTCAGTAGCAGTCTTG) were designed to amplify a 329-bp fragment spanning a 5′ region of the coding sequence. Nuclear run-on transcription experiments (van Blokland *et al.*, 1994) were carried out using hybridization probes for GFP (Reichel *et al.* 1996) and the *Arabidopsis actin-1* gene (M20016).

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ1810/TPJ1810sm.htm

Figure S1. ESTs for an expressed pseudogene in the *WEX* genomic region.

This pseudogene seems to have two transcripts differing at their 3′ end. The shorter transcript was observed in RAFL14-14-A20 (AU235472, AU226180), which starts at 101101 and ends at 99695 on BAC F18A5. The longer transcript was observed in RAFL16-31-E24 (AU237507, AU228581), which starts at 101089, has an undefined section between the two ESTs, and ends at 96820. The *WEX* mRNA starts at 96598 and ends at 94336. Positions of genes are shown as shaded boxes on the top line. Arrowheads in these boxes indicate the direction of transcription. Brackets

indicate ESTs derived from the same clone. The thick arrows indicate a region of very high sequence similarity shared by the pseudogene and *WEX*. Scale bar is 1 kb.

Figure S2. Southern blot analyses of lines 8z2 and 2.

We partially characterized the T-DNA inserts in GFP-transformed lines 2 and 8z2 by Southern blot hybridization using probes for the coding region of *GFP* and *nptII*. The T-DNA region of the expression vector contains two *Hin*dIII sites, one near the right border downstream of the *GFP* gene, and a second one located between the *nptII* and *GFP* genes. The Figure shows that *Hin*dIII-digested DNA prepared from line 2 gave the simple hybridization pattern expected for a single T-DNA insert (Panel B). HindIII-digested DNA prepared from line 8z2 showed a more complex hybridization pattern (Panel A). Based on the number and length of the fragments, we propose that this line has two full-length T-DNA copies arranged in a direct tandem repeat (Panel C). This conclusion was confirmed by sequencing the entire 8z2 locus.

Table S1 *Arabidopsis* RNase D domain proteins

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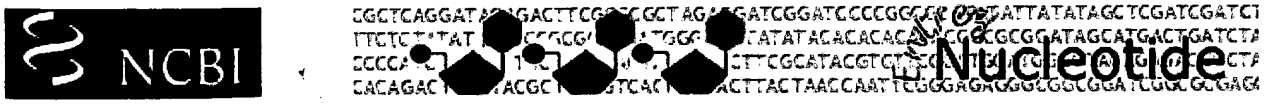

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